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Virus yield parameters in mass production of three Iranian geographic isolates of *Helicoverpa armigera* nucleopolyhedrovirus

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Abstract: Three main parameters of virus propagation, *viz.*, larval stage, inoculation dose and incubation temperature were studied in three nucleopolyhedrovirus isolates of *Helicoverpa armigera*, *i. e.*, Maragheh (MRG), Nebrin (NBN), and Marand (MRD), collected from tomato fields of East-Azarbaijan, Iran, to select the highly promising isolate under laboratory conditions. Among the larval stages evaluated, the early 5th instar larvae recorded the maximum virus yield. The optimum inoculation dose as well as incubation temperature have been determined as 1 965.87 OB/mm^2 and 25℃, respectively. However, the NBN isolate collected from Nebrin recorded the optimum mass production behavior in all the assays.

Key words: *Helicoverpa armigera*; nucleopolyhedrovirus; mass production; yield parameters; inoculation dose; inoculation temperature

1 INTRODUCTION

Mass production of baculoviruses under *in vivo* condition can be easily conducted as a small-scale laboratory process and however it can be economically produced in an industrially level to apply large areas of crops. Standardization of the process is highly supported by culture of host insects on artificial diets, and other microclimatic conditions which could significantly affect the final yield in mass production (Mehrvar, 2011). On the other hand, as viral pathogens are obligate organisms, they have to inevitably multiply on their natural live hosts from which they have been collected (Narayanan, 2003). Carter (1984) listed out the major factors as the host insect, insect diet, insect stage, and virus dosage and incubation which administrate the production of NPVs. As a matter of fact, since *in vitro* propagation of baculoviruses still undertakes to receive increasing attention to come under an economic condition (Shuler *et al.*, 1995), *in vivo* mass production has been found to be the only reasonable method for large scale propagation of virus (Hunter *et al.*, 1998).

Natural variability existing in *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) isolates has to be identified from different geographical regions so the present investigation was undertaken to study the variation in the yield of occlusion bodies (OBs) of different geographical isolates of the virus collected from East-Azarbaijan, Iran, against *H. armigera* larvae to identify the highly promising isolate(s).

2 MATERIALS AND METHODS

2.1 Insects and viruses

The insect culture used in the study was maintained on a semi-synthetic diet based on Shorey and Hale (1965) for culturing *H. armigera* in the Department of Plant Protection, University of Maragheh, Iran. Three Iranian isolates of HearNPV used in this study were collected from tomato fields of three regions of East-Azarbaijan province, Iran, which were MRG from Maragheh region, NBN from Nebrin (Ajabshir) region and MRD from Marand region. These isolates were passaged through early 5th instar larvae of host insect at 25 ± 1℃ to get uniformity in their virulence. All the experiments were performed in insect research laboratory of the Department of Plant Protection, University of Maragheh, and in a facility away from the colony.

2.2 Evaluation of the effect of larval stage on virus mass production

The larval stages used in the study were mid 4th, late 4th, early 5th and mid 5th instars of *H. armigera*. Semi-synthetic diet without formaldehyde was prepared and filled in 5 mL glass vials up to 1/3 height of the vial. Ten μL of virus suspension (5×10^5 OB/larva) was applied onto the diet surface using a micropipette providing a dose of 1 965.78

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OB/mm^2 and then was uniformly spread over the diet surface with the polished blunt end of a glass rod (6 mm). Each larva was weighed individually in an electronic balance and transferred to the treated diet. Three replications were considered for each treatment. A control was included for each stage and isolate also. Each treatment or control had 40 insects. After inoculation, the larvae were incubated at 25 ± 1℃ in an incubator. Observations were recorded at the 24-hour interval and upon death, the cadavers were collected, transferred to sterile vials and the samples were frozen immediately.

Upon work, the cadavers were homogenized individually and transferred to a measuring cylinder and then the volume made up to 25 mL with distilled water. After enumeration of polyhedra the following parameters were calculated:

$$\text{Yield/larva (OB)} = \frac{\text{OB/mL} \times \text{Suspension volume (mL)}}{\text{Total number of cadavers}};$$

$$\text{Yield per 100 inoculated larvae (OB)} = \text{Yield/larva} \times \text{Corrected larval mortality (\%)};$$

$$\text{Productivity ratio} = \frac{\text{Yield/larva}}{\text{OB inoculated/larva}}.$$

2.3 Evaluation of the effct of inoculation dose on virus mass production

Early 5th instar larvae were selected in the study. The exposure doses were 1 965.87, 393.17, and 78.63 OB/mm^2, with the correspounding inculation dose 5×10^5, 1×10^5 and 0.2×10^5 OB/larva, respectively. Experiments were conducted as mentioned above. The mortality was recorded at the 24-hour interval and the experiment was terminated on the 10th day. The cadavers were collected daily, frozen and processed and yield was assessed as described earlier.

2.4 Evaluation of the effect of inoculation temperature on virus mass production

The effect of inoculation temperature on virus yield was studied against early 5th instar larvae which were exposed to the dose of 1 965.87 OB/mm^2 for each viral isolate. Similar procedure was followed as described above except the incubation temperatures which were as room temperature (28 – 31℃), 25 ± 1℃, and 30 ± 1℃.

2.5 Statistical analyses

Data in percentage were transformed to arcsin $\sqrt{\text{percentage}}$ and the data from OB yields were subjected to log transformation and then analyzed. The larval counts were also transformed to $\sqrt{x+0.5}$ values. Analyses of variances (ANOVA) and all mean comparisons (by DMRT) were carried out using SAS software version 6.12. Probit analyses in various experiments were carried out in a Statistical Package for Social Sciences (SPSS).

3 RESULTS

3.1 Effect of larval stage on virus yield

Results showed a significant influence of larval age and weight on the percent larval mortality, yield/larva, yield per 100 inoculated larvae, and productivity ratio of the isolates (Table 1). Among the larval ages evaluated, the early 5th instar larvae showed significantly the maximum yield and productivity. Inoculation of early 5th instar larvae with NBN isolate showed the highest yield/larva of 5.16×10^9 OB, followed by MRG and MRD isolates by the amounts of 4.56×10^9 OB and 3.33×10^9 OB, respectively. The initial larval age and/or weight were critical for obtaining higher OB yield. Also, among the isolates tested in the study, NBN isolate achieved the maximum productivity ratio (1.03×10^4) which was 1.13 and 1.54 times more than MRG and MRD isolates. However, the maximum OB yield was related to the early 5th instar larvae for all the isolates tested in the study (Table 1).

3.2 Effect of inoculation dose on virus yield

Studies on inoculation dose showed an exponential increase in virus production in field with the inoculum dose enhancement. Highest mortality of 81.16, 79.82, and 79.70 percent was recorded at the inoculum dose of 1 965.87 OB/mm^2 of MRG, NBN, and MRD isolates, respectively (Table 2). Likewise, other virus yield parameters, *viz.*, virus yield per larva and for 100 inoculated larvae, were significantly affected at this dose by all the three isolates. The productivity ratios were significantly higher ($P < 0.05$) at the second dose level (393.17 OB/mm^2) with the exception of MRD isolate in which it was higher at the least viral dose (78.63 OB/mm^2) (Table 2). This is because of the initial inoculated viral dose per larva compared to the own yield/larva as per the formula given at section 2.2.

3.3 Effect of incubation temperature on virus yield

The highest virus productivity was acquired for all the isolates when the treated larvae were incubated at 25℃ as compared with that of room temperature and 30℃ (Table 3). The maximum yield/larva, yield per 100 inoculated larvae, and productivity ratios were achieved by NBN isolate, which was followed by MRG and MRD isolates. The productivity was also the highest at 25℃. However, the yield/larva, yield per 100 inoculated larvae, and productivity ratios were the least at 30℃ (Table 3).

Table 1 Effect of larval stage of *Helicoverpa armigera* on the yield of HearNPV isolates at an inoculation dose of 5×10^5 OB/larva and an incubation temperature of 25 ±1℃

HearNPV isolates	Larval instars	Mean harvestable cadavers (out of 40)	Yield/larva ($\times 10^9$ OB)	Yield per 100 inoculated larvae ($\times 10^{11}$ OB)	Productivity ratio ($\times 10^4$)
NBN	Mid 4th	36.95	1.05 d	1.01 c	0.21 d
	Late 4th	36.15	2.64 c	2.54 b	0.53 c
	Early 5th	31.61	5.16 a	4.27 a	1.03 a
	Mid 5th	26.61	3.85 b	2.72 b	0.77 b
MRG	Mid 4th	36.89	0.17 d	0.16 c	0.03 c
	Late 4th	34.75	1.53 c	1.41 b	0.31 b
	Early 5th	30.56	4.56 a	3.68 a	0.91 a
	Mid 5th	27.22	2.22 b	1.62 b	0.44 b
MRD	Mid 4th	36.16	0.50 c	0.48 c	0.10 c
	Late 4th	31.49	0.94 bc	0.79 b	0.19 bc
	Early 5th	29.49	3.33 a	2.66 a	0.67 a
	Mid 5th	25.47	1.56 b	1.08 b	0.31 b

In a column, for each isolate means followed by different letters are significantly different ($P < 0.05$) by Duncan's Multiple Range Test (DMRT). The same for the following tables.

Table 2 Effect of inoculum dose on the yield of HearNPV isolates achieved from the early 5th instar larvae of *Helicoverpa armigera* at an incubation temperature of 25 ±1℃

HearNPV isolates	Exposure dose * (OB/mm^2)	Larval mortality (CM%)	Yield/larva ($\times 10^9$ OB)	Yield per 100 inoculated larvae ($\times 10^{11}$ OB)	Productivity ratio ($\times 10^4$)
NBN	1 965.87	79.82 a	5.24 a	4.18 a	1.05 c
	393.17	79.18 a	4.47 b	3.84 b	4.47 a
	78.63	72.53 b	0.30 c	0.22 c	1.49 b
MRG	1 965.87	81.16 a	4.61 a	3.74 a	0.92 c
	393.17	77.89 b	3.27 b	2.55 b	3.27 a
	78.63	72.24 c	0.28 c	0.20 c	1.39 b
MRD	1 965.87	79.70 a	3.37 a	2.69 a	0.68 c
	393.17	77.25 b	2.49 b	1.92 b	2.49 b
	78.63	71.69 c	1.12 c	0.81 c	5.62 a

* For the exposure doses 1 965.87, 393.17, and 78.63 OB/mm^2, the corresponding inoculation doses are 5×10^5, 1×10^5 and 0.2×10^5 OB/larva, respectively.

Table 3 Effect of incubation temperature on the yield of HearNPV isolates achieved from the early 5th instar larvae of *Helicoverpa armigera* inoculated with 5×10^5 OB/larva

HearNPV isolates	Temperature (℃)	Mean harvestable cadavers (out of 40)	Yield/larva ($\times 10^9$ OB)	Yield per 100 inoculated larvae ($\times 10^{11}$ OB)	Productivity ratio ($\times 10^4$)
NBN	Room *	33.23	3.79 b	3.13 b	0.76 b
	25	32.75	4.99 a	4.00 a	1.00 a
	30	33.08	3.18 b	2.58 c	0.64 b
MRG	Room *	34.23	3.29 b	2.80 b	0.66 b
	25	34.56	4.32 a	3.64 a	0.86 a
	30	32.29	2.66 c	2.25 c	0.53 b
MRD	Room *	34.30	2.29 b	1.95 b	0.46 b
	25	33.01	3.31 a	2.68 a	0.66 a
	30	31.73	1.71 b	1.41 c	0.34 b

* The room temperature ranged 28 – 31℃ during the period of study.

4 DISCUSSION AND CONCLUSION

4.1 Larval stage and virus yield

Larval stage at the time of inoculation has been proved as effective parameter to interfere with the virus yield. Among the larval ages studied, the early 5th instar larvae with a weight range of 66.25 – 68.97 mg recorded significantly the highest yield of 5.16×10^9, 4.56×10^9, and 3.33×10^9 OB/larva in the case of NBN, MRG, and MRD isolates, respectively. Lower OB production registered by mid 4th, late 4th and mid 5th instar larvae (Table 1). Considerable studies had previously stated the same trends on different insects (Teakle and Byrne, 1989; Shieh, 1989; Dhandapani, 1990; Moscardi *et al.*, 1997; Tuan *et al.*, 1998; Monobrullah and Nagata, 2000; Subramanian *et al.*, 2001; Mehrvar, 2011, 2012). Majority of them attained high productivity ratio of virus yield as larval age and weight increased. Actually, the time required for multiplication of the virus in the body of host is mostly related to the age of larvae inoculated. Monobrullah and Nagata (2000) reported that 9-day-old *Spodoptera litura* larvae weighing 125 – 155 mg treated with 4.8×10^6 OB/larva through diet resulted in the maximum productivity of the NPV. Shieh (1989) suggested that from *Helicoverpa zea* larvae with the initial larval weight of 50 – 120 mg, the highest OB yields could be obtained. At this weight range, the larvae could continue their normal growth as that of healthy insects until a day before death resulting in a higher amount of OB yields. Mehrvar (2011, 2012) also observed the same trends between *H. armigera* larval stages. These observations are in agreement with the present findings.

4.2 Inoculation dose and virus yield

Experiments on different inoculation doses of the virus showed significant influence on the percent larval mortality and yield/larva with all the isolates tested. The highest mortality occurred with the highest inoculation dose of 1 965.87 OB/mm^2, recording 81.16, 79.80 and 79.70 percent for the isolates MRG, NBN, and MRD, respectively. These results are in agreement with the findings of Narayanan and Jayaraj (2002) who observed a marked difference between doses as well as a significant interaction between dosages and larval instars. A virus concentration of 3×10^6 OB/mL by diet incorporation technique (Tuan *et al.*, 1998) and 1×10^8 OB/mL by diet surface contamination method (Subramanian *et al.*, 2001; Kumar and Rabindra, 2003) were found to be optimum for *in vivo* production of the virus. However, the productivity ratio progressively decreased as the dose of inoculation increased. This is in partial agreement with the findings by Bell (1991) and Mehrvar (2011) who reported that a lower dose of virus could be used for achieving higher yield.

4.3 Incubation temperature and virus yield

Evaluation of the effect of different incubation temperature on the yield of three HearNPV isolates indicated that the maximum yield/larva was obtained at 25℃ followed by room temperature and 30℃ (Table 3). Likewise, the yield per 100 inoculated larvae and productivity was also highest at 25℃. Similar findings were also reported in the previous studies (McLeod *et al.*, 1977; Stairs, 1978; Johnson *et al.*, 1982; Kelly and Entwistle, 1988; O'Reilly and Miller, 1989; Mehrvar, 2011, 2012). Studies conducted by O'Reilly and Miller (1989) indicated that prolongation in larval growth, even beyond the period of a normal larval stage, would benefit the viral reproduction. Present findings are in agreement with that of previous studies.

Many laboratory studies have demonstrated that nucleopolyhedrovirus is inactivated by exposure to high temperature. McLeod *et al.* (1977) stated that increase in temperature from 15 to 45℃ increased the LD_{50} values of *H. zea* NPV (29.8 – 349.2 OB/mm^2 of diet surface). Stairs (1978) indicated that high temperatures caused direct inactivation of the virus and adversely affected the viral replication. Johnson *et al.* (1982) demonstrated the inhibition of virus activity against the velvet bean caterpillar, *Anticarsia gemmatalis*, at the extremes of temperature of 10 and 40℃. Kelly and Entwistle (1988) found an approximate linear relationship between the *Mamestra brassicae* NPV and the incubation temperature. Histopathological studies by Sathiah (2001) revealed that at 25℃, the growth of fat body in virus-inoculated larvae progressed normally during the early stages of infestation providing adequate substrate for the growth and multiplication of the virus. In *H. armigera* larvae, the virus multiplied at a slow pace at 25℃ allowing the fat bodies to proliferate simultaneously. At higher temperatures the virus multiplied faster and destroyed the fat body before it could grow to provide greater substrate volume. Therefore, a good mass production facility should possess a temperature-controlled incubation chamber to provide a constant temperature of 25 ± 1℃.

As a result, virus mass production *in vivo* in host larvae is the pragmatic method in the world. A variety of parameters have been addressed for

increasing productivity of the HearNPV. Our study revealed that factors like larval age and weight, the inoculum dose, and the incubation temperature could enhance the *in vivo* yield of the virus. Of the larval age evaluated the early 5th instar larvae recorded the maximum yield. The inoculation dose of 1 965.87 OB/mm^2 and the incubation temperature of 25℃ registered the highest *in vivo* virus yield. However, among the isolates tested in this study, NBN isolate collected from Nebrin, East-Azarbaijan, Iran, showed the highest yield in all of the conditions tested compared to the other HearNPV isolates.

References

Bell MR, 1991. *In vivo* production of a nuclear polyhedrosis virus utilizing a tobacco budworm multicellular larval rearing container. *J. Entomol. Sci.*, 26: 69 - 75.

Carter JB, 1984. Viruses as pest control agents. *Biotech. Gen. Engin. Rev.*, 1: 375 - 419.

Dhandapani N, 1990. Studies on The Use of Nuclear Polyhedrosis Virus against *Heliothis armigera* (Hübner) on Cotton and Sorghum. PhD Dissertation, Tamil Nadu Agricultural University, Coimbatore, India.

Hunter FR, Entwistle H, Evans NE, Crook NE, 1998. Formulation. In: Hunter-Fujita FR, Entwistle PF, Evans HF, Crook NE eds. Insect Viruses and Pest Management. John Wiley, New York. 117 - 158.

Johnson DW, Boucias DB, Barfield CS, Allen GE, 1982. A temperature-dependant developmental model for a nuclear polyhedrosis virus of the velvet bean caterpillar, *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.*, 40: 292 - 298.

Kelly PM, Entwistle PF, 1988. *In vivo* mass production in cabbage moth (*Mamestra brassica*) of a heterologous (*Panolis*) and a homologus (*Mamestra*) nuclear polyhedrosis virus. *J. Virol. Methods*, 19: 249 - 256.

Kumar CMS, Rabindra RJ, 2003. Influence of dietary vegetable oils on the tobacco cutworm, *Spodoptera litura* (Fabricius) and its nuclear polyhedrosis virus production. *J. Biol. Control*, 17: 57 - 62.

McLeod PJ, Yearian WC, Young SY, 1977. Inactivation of *Baculovirus heliothis* by ultraviolet irradiation, dew and temperature. *J. Invertebr. Pathol.*, 30: 237 - 241.

Mehrvar A, 2011. Entomopathogenic viruses, mass production technology. In: Borgio JF, Sahayaraj K, Susurluk A eds. Microbial: Principles and Applications. NOVA Science Publishers, USA. 281 - 305.

Mehrvar A, 2012. Studies on the Nucleopolyhedrovirus of *Helicoverpa armigera* (Hübner). Evaluation of Its Geographic Isolates. LAMBERT Academic Publishing, Germany. 422 pp.

Monobrullah MD, Nagata M, 2000. Optimization of *Spodoptera litura* Fab. nucleopolyhedrovirus production in homologous host larvae. *Insect Sci. Appl.*, 20: 157 - 165.

Moscardi F, Leite LG, Zamataro CE, 1997. Production of nuclear polyhedrosis virus of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae): effect of virus dosage, host density and age. *Anais da Sociedade Entomologica do Brasil*, 26: 121 - 132.

Narayanan K, 2003. Microbial control of insect pests using insect viruses. In: Santhakumari P ed. Biological Control of Crop Pests in India. Kalyani Publishers, India. 154 - 175.

Narayanan K, Jayaraj S, 2002. Mass production of polyhedral occlusion bodies of NPV of *Helicoverpa armigera* in relation to dose, age and larval weight. *Indian J. Exp. Biol.*, 40: 846 - 849.

O'Reilly DR, Miller LK, 1989. A baculovirus blocks insect molting by producing ecdysteriods UDP-glycosyl transferase. *Science*, 245: 1110 - 1112.

Sathiah N, 2001. Studies on Improving Production and Formulation of the Nuclear Polyhedrosis Virus of Cotton Bollworm *Helicoverpa armigera* (Hübner). PhD Dissertation, Tamil Nadu Agricultural University, Coimbatore, India.

Shieh TR, 1989. Industrial production of viral pesticides. *Adv. Virus Res.*, 36: 315 - 343.

Shorey HH, Hale RL, 1965. Mass rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.*, 58: 522 - 524.

Shuler ML, Granados RR, Hammer DA, Wood HA, 1995. Overview of baculovirus, insect cell system. In: Shuler ML, Granados RR, Hammer DA, Wood HA eds. Baculovirus Expression Systems and Biopesticides. Wiley, New York. 1 - 11.

Stairs GR, 1978. Effect of wide range of temperature on the development of *Galleria mellonella* to its specific baculovirus. *Environ. Entomol.*, 7: 297 - 299.

Subramanian S, Santharam G, Rabindra RJ, 2001. Optimization of stage and dose of virus inoculum for maximizing the *Spodoptera litura* (Fab.) NPV yield. In: Singh D, Dilawari VK, Mahal MS, Brar KS, Sohi AS, Singh SP eds. Proceedings for Quality Crop Protection in the Current Millennium. Punjab Agricultural University, Ludhiana. 79 - 80.

Teakle RE, Byrne VS, 1989. Nuclear polyhedrosis virus production in *Heliothis armigera* infected at different larval ages. *J. Invertebr. Pathol.*, 53: 21 - 24.

Tuan SJ, Chen WL, Kao SS, 1998. *In vivo* mass production and control efficacy of *Spodoptera litura* (Lepidoptera: Noctuidae) nucleopolyhedrovirus. *Chinese Journal of Entomology*, 18: 101 - 116.

伊朗棉铃虫核型多角体病毒三个分离株在规模化生产中的产量参数

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摘要: 对采自伊朗阿塞拜疆东部西红柿大田的棉铃虫 *Helicoverpa armigera* 核型多角体病毒 3 个分离株[Maragheh (MRG), Nebrin (NBN)和 Marand (MRD)]繁殖的 3 个主要参数(幼虫期、接种剂量和培养温度)进行了研究, 以在室内条件下筛选很有前景的分离株。在测试的各龄幼虫中, 5 龄初期幼虫的病毒产量最高。最适接种剂量和接种温度分别为 1 965.87 OB/mm^{2}和 25℃。而在所有测试中, 采自 Nebrin 的 NBN 分离株在规模化生产中表现最佳。

关键词: 棉铃虫; 核型多角体病毒; 规模化生产; 产量参数; 接种剂量; 培养温度

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